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Metabolic engineering of *Saccharomyces cerevisiae* for lactose/whey fermentation

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Lactose is an interesting carbon source for the production of several bio-products by fermentation, primarily because it is the major component of cheese whey, the main by-product of dairy activities. However, the microorganism more widely used in industrial fermentation processes, the yeast *Saccharomyces cerevisiae*, does not have a lactose metabolisation system. Therefore, several metabolic engineering approaches have been used to construct lactose-consuming *S. cerevisiae* strains, particularly involving the expression of the lactose genes of the phylogenetically related yeast *Kluyveromyces lactis*, but also the lactose genes from *Escherichia coli* and *Aspergillus niger*, as reviewed here. Due to the existing large amounts of whey, the production of bio-ethanol from lactose by engineered *S. cerevisiae* has been considered as a possible route for whey surplus. Emphasis is given in the present review on strain improvement for lactose-to-ethanol bioprocesses, namely flocculent yeast strains for continuous high-cell-density systems with enhanced ethanol productivity.

Introduction

Lactose is a constituent of cheese whey, a major by-product of dairy industries. The lactose can constitute as much as 50 g·L^{-1}. Whey streams could be used as an abundant and renewable raw material for microbial fermentations, with lactose providing the carbon source. In fact, whey disposal has been under consideration for several years, since it is highly polluting and generated in high amounts. Drying is one of the solutions that have been considered and has been implemented on an industrial scale. However, although the discharge problem is solved, no value is added. Whey protein concentrate (WPC) is nowadays one of the major products obtained from cheese whey.[1] When producing WPC, high volumes of a lactose-rich permeate are also generated. This remains a major pollutant and a profitable use for this by-product must be found. The future trend for cheese factories is to move towards zero discharge, i.e., move away from high disposal costs and find more environmentally friendly and profitable applications for lactose.[2] Lactose fermentation to bio-ethanol is one of the possibilities. The use of concentrated lactose solutions (up to 200 g·L^{-1}) is important since it will permit high ethanol titres (up to 10–12% v/v) at the end of fermentation, therefore reducing considerably the ethanol distillation costs. However, natural lactose-fermenting microorganisms, such as the yeast *Kluyveromyces marxianus*, cannot ferment efficiently (i.e., rapidly and with high conversion yields) such high concentrations of lactose. *Saccharomyces cerevisiae* is the organism of choice for bioethanol production. However, this yeast is not able of metabolising the sugar lactose. Thus, strain development programmes through metabolic engineering of *S. cerevisiae* are required for the implementation of lactose-to-ethanol processes with increased productivity.

In 1991 Bailey[3] proposed the emergence of a new discipline called "metabolic engineering", which he defined as "the improvement of cellular activities by manipulations of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology". Initially, metabolic engineering overlapped with applied molecular biology. Developments in genomics and high-throughput system biology tools enhanced the rapid characterization of cellular behaviour, which led to a rapid expansion of metabolic engineering, where strain characterization is often the bottleneck in development programmes. Moreover, advanced genetic engineering techniques along with the sequencing of whole genomes of several organisms and developments in bioinformatics have speeded up the process of gene cloning and transformation.[4] Metabolic engineering processes are categorised typically by the approach taken or the aim.[4] These may be: (1) heterologous protein production (2) extension of substrate range (3) pathways leading to new products (4) pathways for degradation of xenobiotics (5) improvement of overall cellular physiology (6) elimination or reduction of by-product formation, and (7) improvement of yield or productivity. Metabolic engineering of *S. cerevisiae* strains for lactose fermentation fits in the substrate range category of metabolic engineering.

Lactose-Consuming Microorganisms

The number of microorganisms that can use lactose as a source of carbon and energy is limited, yet including bacteria, yeasts and filamentous fungi. Bacteria have evolved different strategies for the uptake and hydrolysis of lactose. The most effective implies the simultaneous phosphorylation and translocation of the sugar across the cell membrane, existing at least two alternative mechanisms for uptake (a lactose-proton symporter and a

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Table 1. *GAL/MEL* genes of *S. cerevisiae* and *GAL/LAC* genes of *K. lactis*

S. cerevisiae		*K. lactis*	
Gene	**Function**	**Gene**	**Function**
Structural/Catabolic genes		**Structural/Catabolic genes**	
MEL1	α-Galactosidase	*LAC12*	Lactose/galactose permease
GAL2	Galactose permease	*LAC4*	β-Galactosidase
GAL1[a]	Bifunctional galactokinase/sensor inducer	*KlGAL1*[a]	Bifunctional galactokinase/sensor inducer
GAL10	Uridine diphosphoglucose 4-epimerase	*KlGAL10*	Uridine diphosphoglucose 4-epimerase
GAL7	Galactose-1-phosphate uridylyltransferase	*KlGAL7*	Galactose-1-phosphate uridylyltransferase
GAL5[b]	Phosphoglucomutase	*KlGAL5*[b]	Phosphoglucomutase
Regulatory genes		**Regulatory genes**	
GAL4	Transcriptional activator	*KlGAL4 (LAC9)*	Transcriptional activator
GAL80	Gal4p repressor	*KlGAL80*	Gal4p repressor
GAL3	Gal80p repressor (sensor/inducer)		

[a]*GAL1* has both catabolic (galactokinase) and regulatory (sensor/inducer) functions. [b]*GAL5* is not specific of the *GAL* regulon, having a more generalised role in carbon metabolism.

lactose-galactose antiporter). Once inside the bacterial cell, the phosphorylated lactose is hydrolysed by a phospho-β-galactosidase (an enzyme that recognises phosphorylated lactose). When the uptake mechanism does not involve phosphorylation, lactose is cleaved intracellularly by a β-galactosidase.[5]

The regulation of lactose utilization by the *lac* operon in *Escherichia coli* has become a paradigm for prokaryotic gene regulation. The *E. coli lacZ* gene (encoding β-galactosidase) has been used commonly as a genetic and biotechnological tool, functioning as a reporter gene for protein expression.[5] Moreover, the *E. coli lacY* gene (encoding lactose permease) was the first gene encoding a membrane transport protein to be cloned into a recombinant plasmid, overexpressed[6] and sequenced.[7] LacYp is a representative for the Major Facilitator Superfamily of transport proteins[8] and its structure has been recently unveiled.[9,10]

Lactic acid bacteria (includes several genera such as Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus) are among the most important lactose-consuming microorganisms, due to their occurrence in milk and dairy products. Besides their food-related significance, the importance of lactic acid bacteria in biotechnology is extended to the production of lactic acid, e.g., from whey fermentation.[11]

Filamentous fungi often utilize lactose at very low rates.[12] In fungi there are two principal alternatives for the catabolism of lactose: (1) extracellular hydrolysis and subsequent uptake of the resulting glucose and galactose monomers and (2) uptake of the disaccharide and subsequent intracellular hydrolysis. Fungi species such as *Aspergillus nidulans*, *Neurospora crassa* or *Fusarium graminearum* follow the second strategy for lactose utilization, while others such as *Hypocrea jecorina* (*Trichoderma reesei*) and *Aspergillus niger* have the ability to secrete β-galactosidase that hydrolyses lactose in the extracellular medium.[12] The yeasts that assimilate lactose aerobically are widespread, but those that ferment lactose are rather rare,[13] including e.g., *Kluyveromyces lactis*, *K. marxianus* and *Candida pseudotropicalis*.

Lactose metabolism in *K. lactis* and the *GAL/LAC* regulon. Similarly to other microorganisms present in milk, *K. lactis* is adapted for the efficient utilization of lactose. The ability of this yeast to metabolise lactose results from the presence of lactose permease and β-galactosidase.[14] The *K. lactis LAC* system is the best studied within the Kluyveromyces genus and is a good model for related species.

The *K. lactis* lactose permease is a membrane protein of 587 amino acids encoded by the gene *LAC12*.[15] The lactose uptake in *K. lactis* is mediated by a transport system inducible by lactose and galactose (the inducer is intracellular galactose).[16] Uptake is mediated by a carrier and is saturated at high substrate concentrations. Dickson and Barr[16] determined a K_m of approximately 2.8 mM for this transport system, while Boze et al.[17] reported a K_m of 1.2–4 mM in a different strain. We have determined recently a K_m of 1.0–1.8 mM for *K. lactis* CBS2359, and our results of lactose uptake by two recombinant *S. cerevisiae* strains expressing the *K. lactis LAC12* gene are also consistent with such K_m values.[18] The transport of lactose in *K. lactis* is an active process, requiring an energy-generating system, which permits the intracellular accumulation of lactose against a concentration gradient.[16,17] The transport is inhibited by the proton ionophore 2,4-dinitrophenol,[16,17] and therefore it has been suggested that the transporter operates, at least in part, by a proton symport mechanism.[16] In other Kluyveromyces species lactose uptake has also been described to proceed via a proton symport mechanism.[19-21] Lac12p shows sequence similarity to the *E. coli* xylose and arabinose proton symporters[15] and a significant sequence and structure homology with the *S. cerevisiae* maltose proton symporter Mal61p,[22] but no significant sequence similarity with the lactose permease (*lacY* gene) of *E. coli*.[15]

The β-galactosidase (lactase) is encoded by the *LAC4* gene[23] and is described to be intracellular.[18,24-26] This enzyme has a K_m for lactose of 12–17 mM and its pH optimum is around 7.[25]

β-galactosidase hydrolyses lactose into glucose and galactose. Intracellular glucose can enter glycolysis while galactose follows the Leloir pathway. In *K. lactis*, the metabolism of lactose and galactose are closely related. The regulatory circuit of the *GAL/LAC* regulon of *K. lactis* (**Table 1**) has been studied in detail

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(reviewed in refs. 24, 27 and 28) particularly in comparison with the *GAL*/*MEL* regulon of *S. cerevisiae* (**Table 1**), which is one of the most intensively studied and best understood genetic regulatory circuits in yeasts and a major model for the study of eukaryotic regulation (reviewed in refs. 29–31).

S. cerevisiae cannot assimilate lactose, yet it can utilise galactose. Some Saccharomyces yeasts can also assimilate melibiose, which is hydrolysed to glucose and galactose by a secretable α-galactosidase encoded by *MEL1*,[32] and other genes of the *MEL* family.[33] Galactose is taken up by a permease, encoded by the gene *GAL2*.[34] Once inside the cell, catabolism of galactose proceeds through the highly evolutionarily conserved Leloir pathway, both in *S. cerevisiae* and in *K. lactis*.[24,29]

Despite the extensive degree of conservation in the group of genes involved in the utilization of galactose between the two yeasts, differences have arisen as a result of their evolution in different environments: *S. cerevisiae* has mainly adapted to glucose, whereas *K. lactis* has adapted to lactose. Therefore, the two yeasts have differences in the modes of regulation that have important consequences in their overall response to carbon sources and may account for major physiological differences between these yeasts.[24,28]

The induction of the *GAL* genes in both *S. cerevisiae* and *K. lactis* is determined by the interplay between three main *GAL*-specific regulatory proteins (**Table 1**): a transcriptional activator (Gal4p, also known as Lac9p in *K. lactis*), a repressor (Gal80p) and a ligand sensor (Gal3p in *S. cerevisiae*; Gal1p in *K. lactis*). This later activates *GAL* gene expression after binding galactose (the inducer) and ATP.[24]

Regulation of *LAC12* and *LAC4* expression in *K. lactis* is controlled by the same mechanisms that regulate *GAL* genes. *LAC12* and *LAC4* are transcribed divergently from an unusually large intergenic region, which works as promoter for the transcription of both genes. The *LAC12-LAC4* intergenic region contains four functional UAS_G elements, which are binding sites for the transactivator Lac9p. Two functional UAS_G elements are located in front of each of the genes at almost symmetrical positions. These elements cooperate in activating transcription of both genes.[35]

Metabolic Engineering of Lactose Consuming/ Fermenting *S. cerevisiae* Cells

One of the first approaches to construct lactose-metabolising *S. cerevisiae* cells consisted in the production of hybrids of *S. cerevisiae* and *K. lactis* or *K. fragilis*, using the protoplast fusion technique.[36-38] The fusant strains were able to ferment lactose and produce more ethanol than the corresponding Kluyveromyces parental strain. More recently, the generation of hybrid strains of *S. cerevisiae* and *K. lactis* able to ferment lactose in sweet and salted whey has been reported.[39,40] The genetic stability of the fusants is a concern when using this methodology.[37]

As aforementioned, some microorganisms are natural lactose consumers, with their lactose metabolisation genes being potential candidates for cloning in *S. cerevisiae* cells in a direct metabolic engineering approach. Predominantly three lactose consumers have been used as sources of lactose genes: the bacteria *E. coli*, the yeast *K. lactis* and the filamentous fungi *A. niger*. Two different strategies can be devised: to clone both the lactose permease and β-galactosidase genes or to direct the β-galactosidase production to the extracellular medium.

Transfer of *E. coli* lactose genes. Three different strategies have been designed to obtain lactose-consuming *S. cerevisiae* cells using *E. coli* lactose genes:

(1) As the β-galactosidase from *E. coli* is cytosolic, lactose has to be transported to the cytoplasm to be hydrolised. Thus, the cloning of the *lacZ* gene alone is not enough to obtain recombinants able to utilize lactose. The functional expression of the *lacY* gene will also be required. Guarente and Ptashne[41] have shown the functional expression of the *lacZ* gene under the regulation of a yeast promoter, which is used widely as a reporter gene. However, when cloning the *lac* operon in a multicopy plasmid in *S. cerevisiae* it was not possible to obtain transformants able to utilize lactose.[42] The yeast transformants, although expressing β-galactosidase, were not able to grow on lactose due to the nonfunctionality of the *E. coli* transport system.

(2) An alternative approach involves the secretion of the *E. coli* β-galactosidase in *S. cerevisiae* cells. In *E. coli*, the attempt to direct β-galactosidase to the membrane using the signal sequence of a membranar protein (lamb) was ineffective.[43] In *S. cerevisiae*, different signal sequences have been tried, namely from the *SUC2*,[44] *MF*α [45] and *STA2*,[46] genes, but these attempts were also unsuccessful. With the *STA2* signal sequence the authors were able to detect 76% of β-galactosidase activity in the periplasmatic space but no enzyme activity was detected in the culture medium. The fusion of glucoamylase residues with *E. coli* β-galactosidase was shown to facilitate its secretion although the secretion was not as efficient as with the glucoamylase gene.[47] However, the authors do not mention if the recombinants were able to grow on lactose. Using the signal sequence of the membranar protein GgpI (the major yeast glycosylphosphatidylinositol-containing protein), it was possible to direct the *E. coli* β-galactosidase to the extracellular medium and for the first time, positive growth on lactose was observed.[48]

(3) The third approach described in the literature deals with the spontaneous lysis of yeast cells overproducing the *E. coli* β-galactosidase enzyme.[49] However, it is worth noting that cell lysis has a negative impact on downstream processing, which represents a disadvantage over the secretion approach. Porro et al.[49] related the release of β-galactosidase activity in the culture medium by recombinant *S. cerevisiae* with the overexpression of the transcriptional activator *GAL4*, which induced partial lysis of the mother cells.[50] Fermentation experiments with these transformants have shown that the release of β-galactosidase in the culture medium was enough to support growth in culture medium containing lactose as the sole carbon source and in whey-based culture medium. Ethanol production was observed in stationary phase with interesting yields (73–84% of the theoretical conversion yield) but unsatisfactory productivities (0.1–0.2 $g \cdot L^{-1} \cdot h^{-1}$) (**Table 2**). Interestingly, diauxic growth was not observed. The authors suggest that an excess of Gal4p may modify the regulatory pathways, leading to a change in cell wall composition, which in turn would be responsible for the lysis of older cells. As Gal4p

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Table 2. Fermentation of lactose to ethanol by recombinant *S. cerevisiae* strains

Characteristics of the strain	Cultivation conditions	Ethanol productivity ($g \cdot L \cdot h^{-1}$)	Ethanol produced ($g \cdot L^{-1}$)	Ethanol yield (%)[a]	Lactose consumed (%)[b]	Reference
Autolytic cells expressing *E. coli lacZ*	Shake-flasks; Yeast Nitrogen Base/Lactose (2–6%)	0.1–0.2	5–18	73–84	>97	49
	Batch/Fed-batch; YP/Lactose (6%) + whey	1.0	9	60–70	100	52
Expression of *K. lactis LAC4* and *LAC12*	Batch; Synthetic lactose (2.2%) medium	0.3	4	34	100	55
	Continuous; Semi-synthetic lactose (5%) medium	10–11	20	70–80	>94	58
	Batch; Cheese whey permeate (10% lactose)	1.8	53	>98	100	63
	Continuous; Cheese whey permeate (5% lactose)	9–10	20	70–80	>98	63
	Batch; Whey powder solution (15% lactose)	0.46	55	70	>98	66
	Batch; Synthetic lactose (15%) medium	1.5–2.0	63	78–84	>98	68
Secretion of *A. niger* β-galactosidase	Anaerobic shake-flasks; Whey permeate (10% lactose)	0.14–0.6	9.7	86	21	76
	Aerobic bioreactor; Synthetic lactose (10%) medium	0.6	30	58	97	76
	Batch; Semi-synthetic lactose (5%) medium	1.0	25	>80	>90	78
	Continuous; Semi-synthetic lactose (5%) medium	9.0	20	74–83	>90	81
	Continuous; Semi-synthetic lactose (10%) medium	3.4–7.0	32–48	70–90	>75	81

[a]Percentage of the theoretical yield, which is 0.538 g of ethanol produced per g of lactose consumed (i.e., 4 mol of ethanol produced per mol of lactose consumed). [b]Percentage of the initial lactose that was consumed during fermentation.

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is involved in glucose repression of galactose-utilizing genes, the excess of Gal4p could also be responsible for the simultaneous metabolisation of glucose and galactose by the transformants.[49] Compagno et al.[51] reported the use of *S. cerevisiae* cells expressing the *lacZ* gene permeabilized by toluene in the bioconversion of lactose/whey to fructose diphosphate. Compagno et al.[52] have crossed the *S. diastaticus* yeast strain JM2099 (having glucoamylase activities allowing a partial hydrolysis of starch) with the laboratory strain W303 to develop a strain able to grow simultaneously on starch and whey/lactose. Haploid cells able to grow on starch and bearing the appropriate mutation (i.e., *leu*2) have been isolated and transformed with the plasmid pM1 (previously used in the generation of lactose-utilizing yeast strains[49]). In this way, a yeast strain for the simultaneous utilization of lactose and starch has been developed.

Transfer of *K. lactis* lactose genes. The utilization of lactose by Kluyveromyces strains is based on a lactose transport system together with an intracellular β-galactosidase (see above). Hence, the same three strategies used with the *E. coli* lactose metabolising genes have also been used with the Kluyveromyces *LAC* genes. However, as the lactose transport system from Kluyveromyces is eukaryotic, it is more prone to work in the phylogenetically related *S. cerevisiae* than the *E. coli* one. Indeed, using simultaneous expression of the lactose permease and intracellular β-galactosidase it was possible to obtain *S. cerevisiae* cells growing on lactose.[53-56]

Sreekrishna and Dickson[53] were the first to construct Lac$^+$ *S. cerevisiae* strains by transfer of the *LAC12* and *LAC4* genes of *K. lactis*. A 13 kb region of the *K. lactis* genome, comprising the two genes and their intergenic region, was used in the construction. Thus, transcriptional expression of the genes was controlled by the endogenous *K. lactis* promoter (see above). These authors have only obtained Lac$^+$ transformants when using indirect selection (first selected for G418 resistance and then for growth on lactose). Moreover, they reported that the Lac$^+$ transformants had integrated 15–25 tandem copies of the vector containing the *LAC* genes into a host chromosome. The transformants obtained presented a slow growth phenotype in lactose medium (doubling time in lactose minimal media of 6.7 h).[53]

Jeong et al.[54] have constructed the plasmid SH096 by isolating the *K. lactis* lactose-utilizing genes, including *LAC4* and *LAC12*, and cloning that DNA into a yeast integrative vector. The yeast strains transformed with this vector grew weakly on minimal lactose medium.[54]

More recently, Rubio-Teixeira et al.[55] cloned the *LAC4* and *LAC12* genes in *S. cerevisiae* but their strategy involved placing *LAC* genes under the control of the *CYC-GAL* promoter (a galactose-inducible hybrid promoter) and targeting genomic integration to the ribosomal DNA region (*RDN1* locus). The Lac$^+$ transformants were selected in culture medium containing lactose as the sole carbon source. However, the transformants grew slowly in lactose while being stable mitotically. The transformants were then crossed with wild-type strains, yielding meiotic segregants with good growth and lactose assimilation capacity. Finally, two selected haploids were mated to generate a fast-growing Lac$^+$ diploid strain. This strain exhibited a respiro-fermentative metabolism similar to that of *K. lactis*, with high biomass yield but low ethanol production[55] (**Table 2**). The same approach was used to construct Lac$^+$ baker's yeast[57] with the ability to metabolise lactose. Growth of the new strain on cheese whey affected neither the quality of bread nor the yeast gassing power.[57]

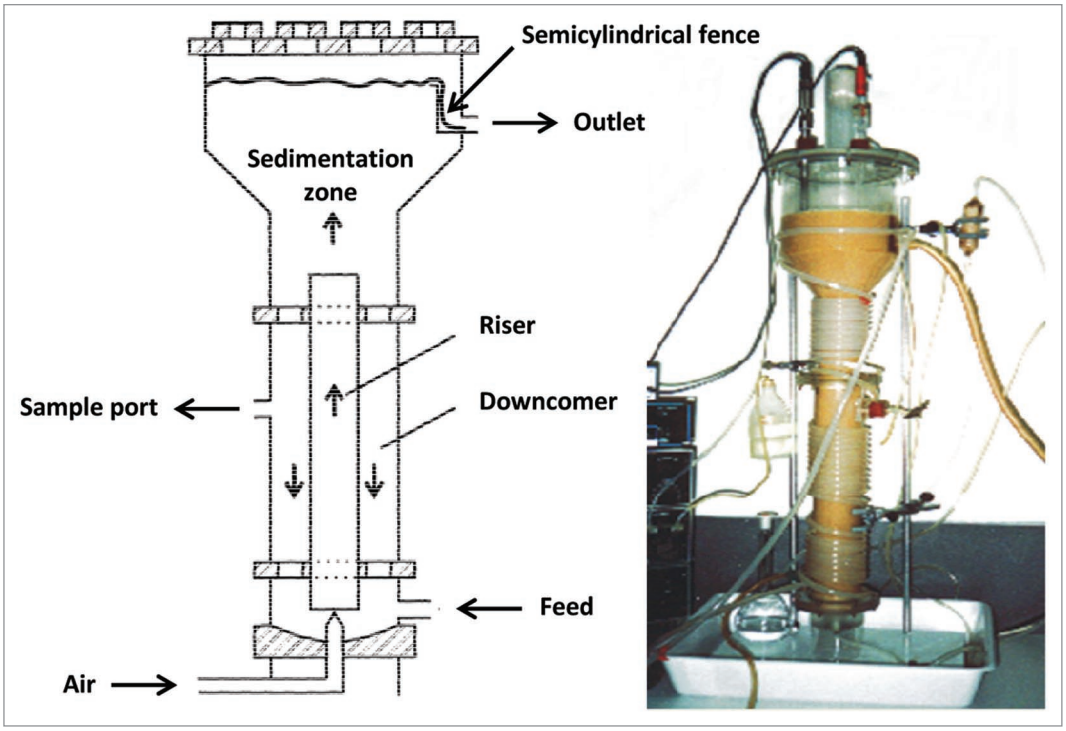

Figure 1. Schematic representation and photography (credits: Lucília Domingues) of the airlift bioreactor.

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In our laboratory, a flocculent *S. cerevisiae* Lac$^+$ strain was constructed[56] using the same plasmid (pKR1B-LAC4-1) employed by Sreekrishna and Dickson[53] but with a different selection procedure. The plasmid KR1B-LAC4-1 was co-transformed with a linear fragment of the plasmid YAC4 (containing the *URA3* gene) into an *ura*$^-$ strain (*S. cerevisiae* NCYC869-A3). Selection was done for *ura*$^-$ complementation in minimal medium plates containing galactose as carbon source. Xgal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) was included in the plates, allowing the identification of clones with β-galactosidase activity (blue colonies). Only 4 blue colonies were obtained (out of 1212) and only 2 kept a stable Lac$^+$ phenotype. One of these transformants exhibited unusual morphology and pseudomycelium and therefore was rejected. The other (named *S. cerevisiae* NCYC869-A3/T1, or simply T1) was selected for more detailed characterization. Surprisingly, the recombinant strain metabolised the same amount of lactose (10 g·L^{-1}) regardless of the initial lactose concentration and ethanol production was very low. Moreover, the doubling time of the recombinant strain in lactose minimal medium was 5 h.[56] After an adaptation period, where the strain was maintained in periodically-refreshed liquid lactose medium, the performance of the recombinant strain in lactose culture medium was significantly improved and an ethanol conversion yield close to the theoretical value could be obtained. T1 kept the plasmid pKR1B-Lac4-1 in its autonomous form contrary to the Lac$^+$ transformants obtained by Sreekrishna and Dickson.[53] The recombinant T1 presented different flocculation behaviour from the host strain *S. cerevisiae* NYCY869-A3 although the recombinant strain was able to flocculate. The flocculation phenotype of the recombinant strain was more sensitive to environmental conditions than that of the host by losing its ability to flocculate more easily. After the adaptation period referred above, it was observed that this recombinant strain metabolised 50 g·L^{-1} lactose in less than 40 h, producing 16 g·L^{-1} ethanol.[56]

These preliminary results indicated that the recombinant strain could be used in a continuous high-cell-density fermentation system if the flocculation instability could be overcome.[58] Continuous operation in a bioreactor with an appropriate design[59] can be used to select for the most flocculating cells from a mixed culture, allowing for the possibility of accumulating a high biomass concentration in the bioreactor with the inherent advantages of operating as a continuous high-cell-density system.[60] The choice of using an airlift bioreactor, which exhibits low shear stresses due to the absence of mechanical agitation,[61] enabled the selection for the most flocculating yeast cells. The non-flocculent cells were washed-out from the bioreactor and the existence of a sedimentation zone in the top of the reactor coupled with the semicylindrical fence in the outlet region helped in the retention of the cells with higher sedimentation ability (**Fig. 1**). Selection of a 100% flocculent culture was achieved within just 13 days of continuous operation. For the continuously operating bioreactor, an ethanol productivity of 11 g·L^{-1}·h^{-1} (corresponding to a feed lactose concentration of 50 g·L^{-1} and a dilution rate of 0.55 h^{-1}) was obtained (**Table 2**), which is 7 times larger than the continuous conventional systems.[58] The system stability was confirmed by keeping it in operation for 6 months.[58] Also, the resistance of this system to nonflocculent contaminants was proved by artificially contaminating the bioreactor operating at 0.45 h^{-1} dilu-

tion rate with a 1 x 10^7 cells·mL^{-1} culture of recombinant *E. coli* expressing GFP (Green Fluorescent Protein).[62]

When operating in continuous high-cell-density system using cheese whey permeate as substrate an ethanol productivity near 10 g·L^{-1}·h^{-1} (corresponding to 0.45 h^{-1} dilution rate) was obtained[63] (**Table 2**). While producing ethanol, the recombinant *S. cerevisiae* strain cleared the cheese whey permeate of most organic substances, allowing for a significant reduction in the pollutant load of cheese whey. The use of two-fold concentrated cheese whey permeate was also considered, resulting in a fermentation product with 5% (w/v) ethanol.[63] However, it was not possible to operate continuously using the high-cell-density airlift bioreactor with concentrated cheese whey due to a deflocculating effect attributed to the salts concentration.[63] A hydrodynamic and rheological analysis of the continuous airlift bioreactor operating at high-cell-density with the recombinant T1 strain was conducted.[64] Measurements of liquid circulation velocity revealed a critical value of biomass concentration at which a dramatic deceleration of net liquid flow appeared with increasing biomass quantity. Rheological analysis demonstrated an exponential increase in viscosity of the yeast floc suspension at the same biomass concentration (around 73 g·L^{-1}) corresponding to 42.8% v/v of solid fraction.[64] A multi-route, non-structural kinetic model was developed for interpretation of ethanol fermentation of lactose using the recombinant flocculent T1 strain.[65] In this model, the values of different metabolic pathways were calculated applying a modified Monod equation rate in which the growth rate is proportional to the concentration of a key enzyme controlling the single metabolic pathway. Three main metabolic routes for *S. cerevisiae* were considered: oxidation of lactose, reduction of lactose (producing ethanol), and oxidation of ethanol. A very good agreement between experimental data and simulated profiles of the main variables (lactose, ethanol, biomass and dissolved oxygen concentrations) was achieved.[65]

Unexpectedly, the strain lost its improved phenotype after storage at -80°C. Thus, another adaptation period was required for the already adapted culture of T1 that had been kept at -80°C.[56] With the objective of obtaining a stable recombinant that could be used industrially, a long-term evolutionary engineering experiment was conducted and a stable evolved strain was obtained and named T1-E.[66] We identified two molecular events that targeted the *LAC* construct in the evolved strain: a 1,593-bp deletion in the intergenic region (promoter) between *LAC4* and *LAC12* and a decrease of the plasmid copy number by about 10-fold compared to that in the original recombinant. Moreover, we have compared the transcriptomes of the original and the evolved recombinant strains growing in lactose, using cDNA microarrays. Microarray data revealed 173 genes whose expression levels differed more than 1.5-fold.[67] About half of these genes were related to RNA-mediated transposition and the others were genes involved in DNA repair and recombination mechanisms, response to stress, chromatin remodeling, cell cycle control, mitosis regulation, glycolysis and alcoholic fermentation.[67] The evolved T1-E strain retained improved lactose fermentation and enhanced flocculation phenotype even after -80°C storage. Strain T1-E was able to ferment efficiently high concentrations of lactose to ethanol, producing a maximum of 8% (v/v) ethanol from mineral medium with 150 g·L^{-1} lactose[68] (**Table 2**). It was also capable of fermenting three-fold concentrated cheese whey, containing 150 g·L^{-1} lactose, yielding an ethanol titre of 7% (v/v)[66] (**Table 2**).

Finally, the use of thermosensitive autolytic mutants has been reported in order to release Kluyveromyces β-galactosidase into the culture medium.[69,70] Recombinant *S. cerevisiae* strains that are able of secreting *K. lactis* β-galactosidase have also been constructed.[71,72] These approaches were used with the aim of developing a system for *K. lactis* β-galactosidase production and not for lactose bioconversion to ethanol. More recently, and with the same aim, a hybrid protein between *K. lactis* and *A. niger* β-galactosidase was constructed that increased the yield of the recombinant protein released to the growth medium.[73]

Transfer of *A. niger* lactose genes. The filamentous fungi *A. niger* is an efficient producer of several secreted glycoproteins, some of which are used in industrial processes. Among these is β-galactosidase, mainly used to hydrolyse lactose in acid whey.[74] The cloning of the *lacA* gene (coding for *A. niger* β-galactosidase) with its own signal sequence resulted in recombinant *S. cerevisiae* cells secreting β-galactosidase.[75-78]

Kumar et al.[75] have obtained *S. cerevisiae* cells growing on lactose by transforming the cells with a yeast multicopy expression vector carrying the cDNA for *A. niger* secretory β-galactosidase under the control of *ADH1* promoter and terminator. Ramakrishnan and Hartley[76] studied the fermentation properties of the transformants and transformed polyploid distiller's yeast (Mauri) with the same vector. Diauxic growth patterns were not observed for the transformants growing on lactose while a typical biphasic growth was observed on a mixture of glucose and galactose under aerobic and anaerobic conditions. Rapid and complete lactose hydrolysis and higher ethanol (0.31 g per g of sugar) and biomass (0.24 g per g of sugar) production were observed with distiller's yeast grown under aerobic conditions.[76] However, plasmid stability was low.

In our laboratory, flocculent *S. cerevisiae* strains secreting β-galactosidase were constructed[77,78] using the vector developed by Kumar et al.[75] Optimization of bioreactor operation together with culture conditions (lactose and yeast extract concentration) led to a 21-fold increase in the extracellular β-galactosidase produced when compared with preliminary shake-flask fermentations.[79] To improve the genetic stability of the strains the *lacA* gene expression cassette was targeted to the δ-sequences in the genome.[80] Even though our main goal was to produce heterologously *A. niger* β-galactosidase, we have observed that these strains produced ethanol from lactose/whey with close to theoretical yields in batch[78] and in high-cell-density continuous fermentations[81] with complete lactose utilisation (**Table 2**). The use of this strain in the dairy industry is very attractive for the simultaneous production of ethanol and β-galactosidase and the reduction of the organic load of the whey. The recombinant enzyme can be used for the generation of other products within the dairy industry (e.g., lactose-free products, hydrolysed whey syrups).

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Conclusions

For the conceivable future, there will be large surplus of whey and whey permeates worldwide. There is not one single solution to the problem of excess whey. The dairy industry should explore further the new possibilities for lactose as a raw material for processing in food and especially non-food industries.[2] The development of processes and products for high volume markets will determine if a larger utilization of the lactose present in cheese whey is possible. Lactose-to-ethanol processes may be one of the solutions. Genetic engineering approaches have been used for the last 25 years with the aim of developing *S. cerevisiae* strains for such processes. Different strategies have been employed, as reviewed here, namely using the lactose metabolisation genes from the bacteria *E. coli*, the yeast *K. lactis* and the filamentous fungi *A. niger*. When considering the metabolic engineering of *S. cerevisiae* cells for lactose-to-ethanol bioprocesses, the best results have been obtained with recombinants constructed with the *K. lactis* genes. Nevertheless, the direct cloning of *LAC4* and *LAC12* from *K. lactis* with its own promoter did not allow the direct selection of transformants in lactose plates and resulted in recombinants with a slow growth phenotype in lactose medium. When using other yeast promoters, direct selection of transformants was possible but the slow growth phenotype was still observed. This indicates that the cloning of the *LAC4* and *LAC12* genes per se is not enough to obtain a good lactose growth phenotype. Thus, the crossovers with wild type strains or evolutionary engineering approaches were needed for the successful generation of efficient lactose-consuming recombinants. In the near future, further metabolic engineering efforts combined with improved bioprocess design will drive the development of more efficient fermentation processes for the conversion of concentrated whey to bioethanol. Furthermore, the simultaneous production of multiple commodities from whey (for instance β-galactosidase and ethanol) will improve the economics of whey fermentation processes.

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